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The IGF axis is known to play an important role in the epidemiology of many tumors. IGFBP-3 promotes apoptosis in cancer cells by both IGF-dependent and -independent mechanisms. We have previously shown that IGFBP-3 is rapidly internalized and localized to the nucleus, where its interactions with the nuclear receptor RXRα are important in apoptosis induction. We demonstrate that phosphorylation of IGFBP-3 (S156) by DNA-PK enhances its nuclear accumulation, and is essential for its ability to interact with RXR and induce apoptosis in cultured prostate cancer cells. Indeed, IGFBP-3-S156A is completely unable to induce apoptosis in 22RV1 cells. Using specific chemical inhibitors, we investigated the contribution of other protein kinases to the regulation of IGFBP-3-induced apoptosis. Preventing the activation of CK2 enhanced the apoptotic potential of IGFBP-3. We mapped two potential CK2 phosphorylation sites in IGFBP-3: S167 and S175. These sites were mutated to Ala, and the resulting constructs were transfected in to LAPC4 and 22RV1 prostate cancer cells. WtIGFBP-3 and IGFBP-3-S175A induced apoptosis to a comparable extent; however, IGFBP-3-S167A was far more potently apoptosis-inducing. These effects were specific to apoptosis-induction, however, since wtIGFBP-3 and IGFBP-3/S167A had comparable effects on cell growth and proliferation, assessed by MTT and BrdU incorporation assays. Interestingly, IGFBP-3-S167A was able to induce apoptosis even in the absence of active DNA-PK, while the DNA-PK non-phosphorable IGFBP-3-S156A mutant regained the ability to induce apoptosis when CK2 activity was inhibited chemically or by using siRNA. Together, these data reveal two key regulatory phosphorylation sites in the central region of IGFBP-3. Phosphorylation of S156 by DNA-PK promotes apoptosis, whilst phosphorylation of S167 by CK2 limits the ability of IGFBP-3 to induce apoptosis. Interestingly, our data suggest that the anti-apoptotic phosphorylation event induced by CK2 is dominant. Pre-treatment of 22RV1 cells with IGFBP-3-siRNA also limits the ability of high doses of CK2 inhibitor to induce apoptosis. These effects can be reversed by the addition of exogenous IGFBP-3 protein, suggesting that inhibition of IGFBP-3 action by CK2 may be a key mechanism through which CK2 induces cell survival. These studies reveal multi-site phosphorylation of IGFBP-3 that both positively and negatively regulate its apoptotic potential. Understanding such intrinsic regulation of IGFBP-3 action may enhance the development of potential cancer therapies.

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Introduction

The actions of IGF-I and -II are regulated by a family of six high affinity binding proteins, of which IGFBP-3 is the most abundant serum, where it forms a ternary complex with acid labile subunit (ALS) and IGF [1]. In addition to its role in regulating IGF action, IGFBP-3 exerts many IGF-independent effects to inhibit cell proliferation and enhance apoptosis in many cell types, including prostate [2] and breast [3,4,5] cancer. However, little is understood about the cellular mechanisms regulating IGFBP-3 action. IGFBP-3 is subject to post-translational modifications such as glycosylation and proteolysis, and also contains consensus phosphorylation sites for a variety of protein kinases. We investigated the significance of phosphorylation for the cellular actions of IGFBP-3 in prostate cancer. We identify phosphorylation to be a critical regulatory step in the growth-inhibitory and apoptosis-promoting actions of IGFBP-3.

Body

Statement of Work Task 1

Several DNA constructs have been made in which various phosphorylation sites of IGFBP-3 have been mutated to Alanine (table 1). Additionally, constructs of IGFBP-3 with other key sites mutated, such as the nuclear localization signal, and with the signal peptide removed have also been created. Site-directed mutagenesis was carried out using a 2-step PCR reaction based on a method published by Adereth *et al*. [6], Fig 1.

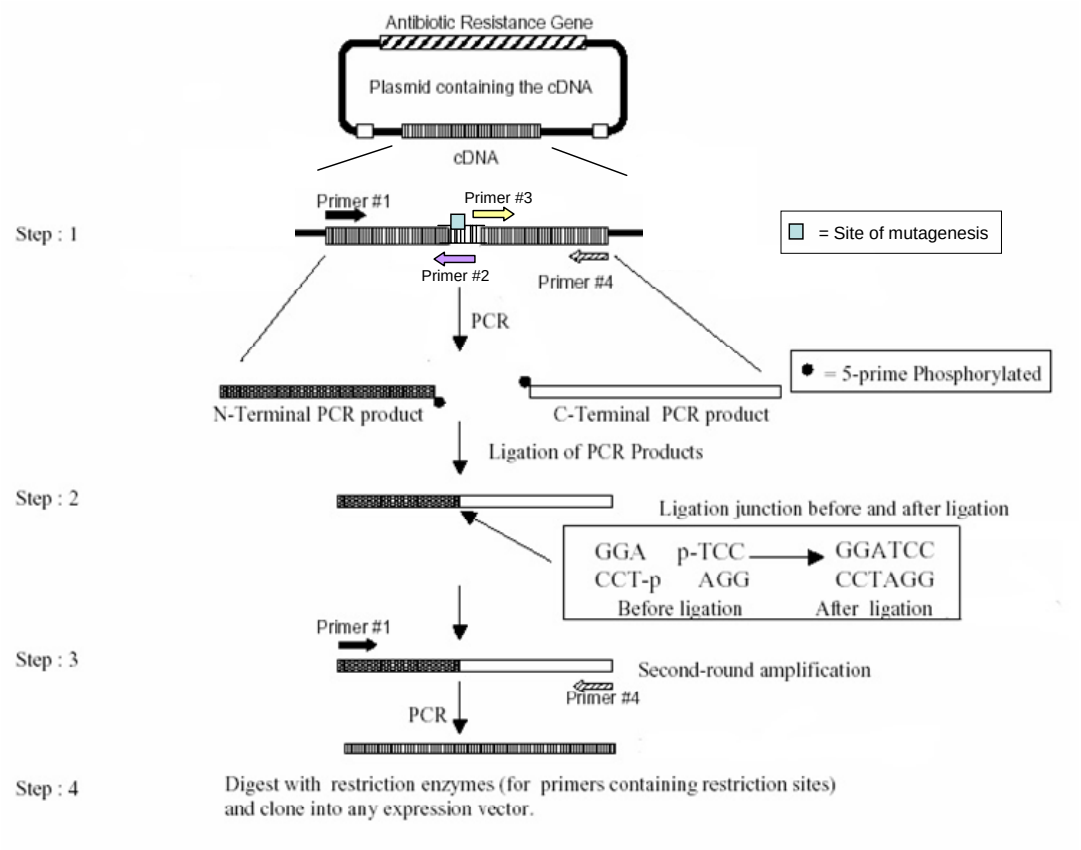

Figure 1 – Strategy for site directed mutagenesis (adapted from Adereth *et al*). Step 1- Introduction of a specific mutation as an example. Step 2 - Ligation of the PCR products. Step 3 - Amplification of the ligated product to generate cDNA containing the desired mutation. Step 4 - Restriction digestion of the final PCR product and ligation into the expression vector.

Mutants were cloned in to a pCMV-FLAG vector (Sigma), and were verified by DNA sequencing (Laragen). The transfection efficiency of all constructs in to both LAPC4 and 22RV1 prostate

cancer cells was optimized using GeneJuice transfection reagent (Novagen) and verified by immunoblotting for the FLAG-tag.

IGFBP-3 Mutant	Altered function
S165A	Putative DNA-PK phosphorylation site
T170A	Putative DNA-PK phosphorylation site
S156A	DNA-PK phosphorylation null
S156E	DNA-PK constitutive phosphorylation
S175A	Putative CK2 phosphorylation site
S167A	CK2 phosphorylation null
S111A/S113A	Previously published phosphorylation sites
nsIGFBP-3	Non-secreted IGFBP-3
NLS-IGFBP-3	No nuclear localization sequence

Table 1 – Mutants of IGFBP-3 generated

Statement of Work Task 2

We have investigated the relevance of DNA-PK phosphorylation for IGFBP-3 action in different prostate cancer cell systems, as described in the statement of work task 2. For detailed descriptions of the role of DNA-PK phosphorylation for IGFBP-3 action, please refer to our manuscript recently published in Cancer Research [7]. Briefly, we use chemical inhibitors and DNA-PK null cell lines to reveal that in the absence of active DNA-PK, IGFBP-3 has reduced nuclear localization and is unable to interact with its nuclear binding partner RXRα. It is also unable to exert its growth inhibitory or apoptosis-inducing actions in LAPC4 or 22RV1 prostate cancer cells. We confirmed potential DNA-PK phosphorylation sites by bioinformatics as S156, S165 and T170. By site-directed mutagenesis and transfection experiments, we identified S156 as the amino acid specifically phosphorylated by DNA-PK. Consequently, IGFBP-3/S156A has impaired nuclear localization, and is unable to induce apoptosis in either 22RV1 or LAPC4 prostate cancer cells. These studies led to the hypothesis that the phosphorylation of S156 of IGFBP-3 by DNA-PK causes a conformational change which allows its interaction with RXRα and the subsequent induction of apoptosis.

Once a clear role for DNA-PK had been identified in the regulation of IGFBP-3-induced apoptosis, the potential role of other protein kinases in regulating IGFBP-3 action was investigated.

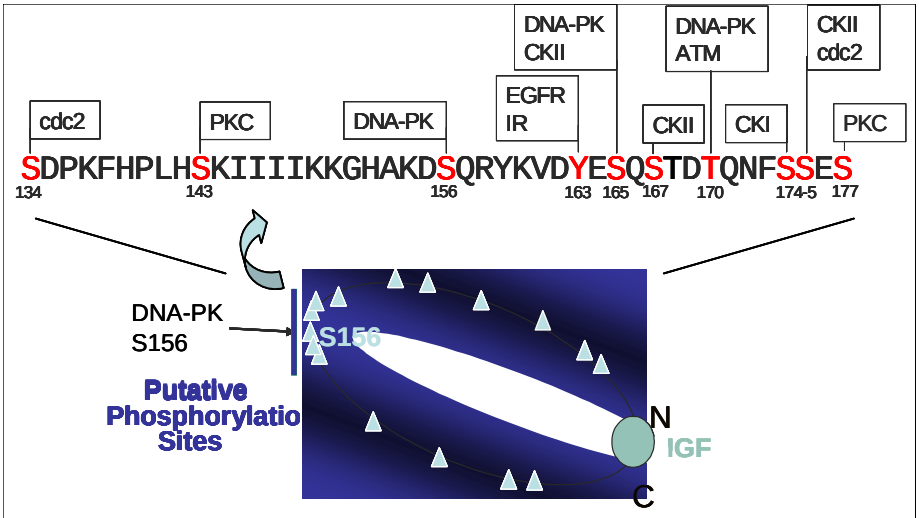

Figure 2 - Cartoon demonstrating the phosphorylation sites identified in the region surrounding S156 of IGFBP-3. Putative phospho-acceptor residues are colored red, and potential kinases are demonstrated in boxes.

Because our hypothesis for the regulation of IGFBP-3 action involves a conformational change which may regulate its intracellular localization, we focused on the central region of IGFBP-3 surrounding S156 when considering other potential phosphorylation sites. When this region is studied using different bioinformatics programs [8], multiple putative phosphorylation sites for a variety of different protein kinases can be identified (Fig. 2).

To identify which, if any, of these putative phosphorylation sites may be phosphorylated and play a role in regulating IGFBP-3 action, we purchased chemical inhibitors (where available) against GSK-3 (BIO, EMD Biosciences), CKII (TBB, EMD Biosciences), PKC (Myr-PKC [19-27] PTK, Biosource) and EGFR (CPCA, EMD Biosciences). We then compared the ability of IGFBP-3 to induce apoptosis in prostate cancer cells in the presence of each specific chemical inhibitor (Fig. 3). Methodology for analysis of apoptosis was carried out as described previously (CR ref). Treatment of 22RV1 with 1 μg/ml IGFBP-3 significantly induced apoptosis, as previously observed. Incubation with 100 μM Myr-PKC [19-27] slightly, but not significantly, induced apoptosis. Co-incubation of 22RV1 cells with Myr-PKC [19-27] and IGFBP-3 led to an additive apoptosis induction (Fig. 3A), although the difference between the apoptosis induced by IGFBP-3 in the presence and absence of the chemical inhibitor was not statistically significant. Similarly, inhibition of GSK-3 activity with 25 μM BIO had no effect on IGFBP-3-induced apoptosis (Fig. 3B). Interestingly, inhibition of CK2 or EGFR activity by incubation with 900 nM

TBB or 10 μM CPCA, respectively, actually enhanced the ability of IGFBP-3 to induce apoptosis (Fig. 3C & D). These initial experiments revealed that other kinases indeed may play a role in the regulation of IGFBP-3-induced apoptosis, and that their manipulation may facilitate the development of more bioactive forms of IGFBP-3.

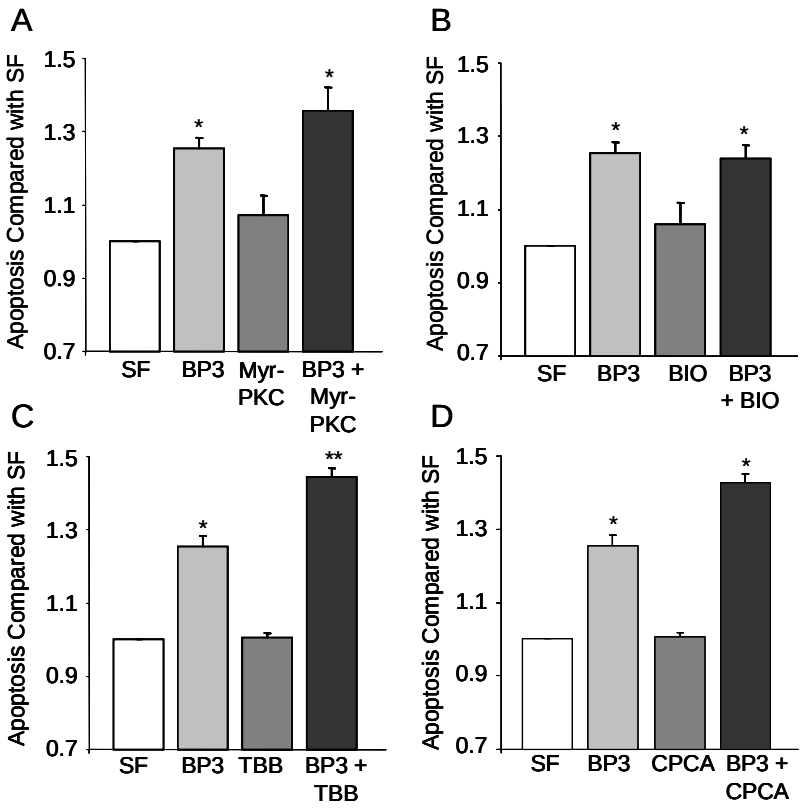

Figure 3 – Effect of kinase inhibition on IGFBP-3-induced apoptosis. 22RV1 cells were incubated for 24 h in SF media in the presence or absence of 1μg/ml IGFBP-3 ± 100 μM Myr-PKC [19-27] (PKC inhibitor, *A*), 25 μM BIO (GSK-3 inhibitor, *B*), 900 nM TBB (CK2 inhibitor, *C*) or 10 μM CPCA (EGFR inhibitor, *D*). Apoptosis was assessed by ELISA for fragmentation of histone-associated DNA. Data are presented as means ± S.E.M. Significance than mean is different from 1: **P*<0.05, ***P*<0.01.

CK2 (formerly casein kinase 2) is a highly conserved, ubiquitously expressed kinase which plays a key role in the regulation of cell growth, proliferation and apoptosis. Importantly, CK2 is dysregulated in most cancers, including prostate [9]. We therefore decided to further investigate the potential relevance of CK2-mediated phosphorylation of IGFBP-3.

To elucidate the significance of CK2 phosphorylation in the regulation of IGFBP-3-induced apoptosis, we utilized two chemical inhibitors against CK2, TBB and DMAT, in addition to siRNA against CK2α. Initial experiments confirmed that incubation of IGFBP-3 with low doses of either inhibitor (900 nM and 100 nM, respectively), insufficient to induce apoptosis, was adequate to reduce the serine phosphorylation of IGFBP-3, as demonstrated by immunoprecipitation of IGFBP-3 followed by phospho-serine-specific immunoblotting (Fig. 4A). Confirming the preliminary data obtained in Fig. 3C, we demonstrated that when CK2 activity is inhibited by either chemical inhibitor, exogenously added IGFBP-3 had significantly enhanced ability to induce apoptosis in both 22RV1 and LAPC4 prostate cancer

cells, as determined by caspase-3/-7 activity (Fig. 4B). These data were confirmed using siRNA against CK2α. Transfection of LAPC4 cells with a verified CK2α duplex of siRNA (STEALTH, Invitrogen) resulted in CK2α protein levels which were reduced by approximately 75%, as determined by immunoblotting (Fig. 4C). IGFBP-3 exogenously added to cells which had been transfected with CK2α siRNA had an ability to induce apoptosis which was significantly greater compared with IGFBP-3 which had been added to cells transfected with scrambled siRNA control (Fig. 4D).

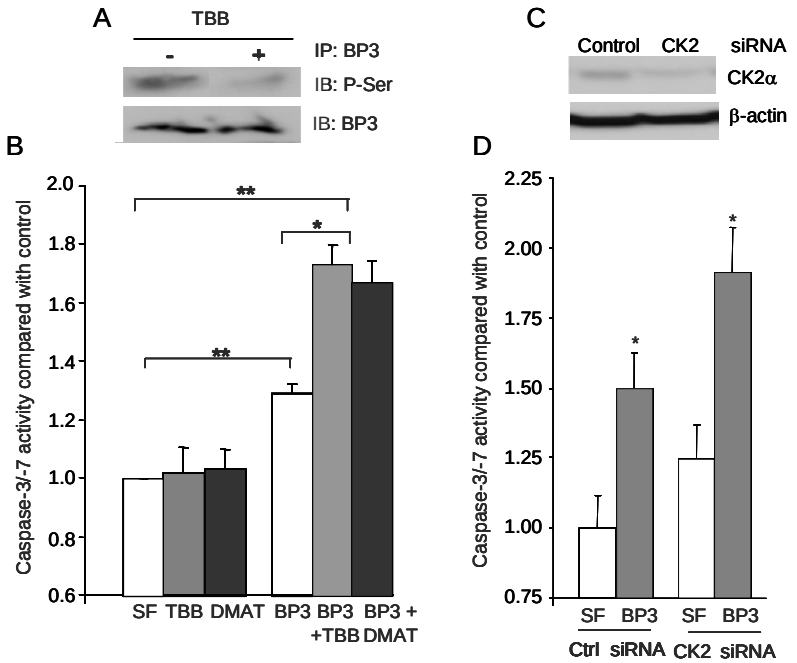

Figure 4 – Inhibition of CK2 activity enhances the apoptotic potential of IGFBP-3. *A,* LAPC4 cells were incubated with 900 nM TBB or 100 nM DMAT in serum free media for 24 h. The phosphorylation status of IGFBP-3 was assessed by immunoprecipitation of IGFBP-3 followed by immunoblotting for P-Ser and IGFBP-3. *B,* Apoptosis was assessed in LAPC4 cells treated as in *A* by caspase-*3*/-7 activity after the additional incubation for 24 h with 1 μg/ml IGFBP-3. *C,* LAPC4 cells were transfected with CK2α or control (scrambled) siRNA and incubated for 72 h. Reduced protein expression of CK2α was confirmed by immunoblot for CK2α or β-actin (loading control) *D*, Caspase-3/-7 activity by cleavage of a luminescent substrate was assessed in cells transfected as in *C* after incubation for 24 h in SF media with 1 μg/ml IGFBP-3. Significance that mean is different from 1: * $P<0.05$; ** $P<0.01$

Since we identified that the intracellular localization of IGFBP-3 is regulated by its phosphorylation by DNA-PK, we investigated whether the enhanced apoptosis induction observed with reduced CK2 activity correlated with enhanced nuclear localization of IGFBP-3. LAPC4 cells were incubated with 900 nM TBB for 24 hours, and cytoplasmic and nuclear fractions were isolated using the NUClear fractionation kit (Sigma-Aldrich). The intracellular localization of IGFBP-3 was assessed by SDS-PAGE followed by immunoblotting, and the validity of cytoplasmic and nuclear fractions was confirmed by Hsp60 and TBP expression, respectively. Consistent with the nuclear localization of IGFBP-3 being critical for apoptosis induction in our cell systems, enhanced nuclear localization of IGFBP-3 was observed when CK2 activity was inhibited (Fig. 5).

In 22RV1 and LAPC4 cells, IGFBP-3 has been shown to require nuclear localization in order to activate the intrinsic apoptotic pathway. However, the mechanism of action of IGFBP-3 for apoptosis induction remains controversial. Others have suggested the existence of a putative cell surface receptor through which IGFBP-3 activates caspase-8 and the extrinsic apoptotic pathway [4], while others have suggested that, in PC-3 cells, the nuclear localization of IGFBP-3 and its interaction with RXRα are not required for apoptosis induction [10]. We therefore compared the effect of inhibiting DNA-PK and CK2 on IGFBP-3 action in PC-3, LAPC4 and 22RV1 cells. As previously demonstrated, IGFBP-3 induces apoptosis in 22RV1 and LAPC4 cells that is inhibited by incubation with DNA-PK inhibitor and enhanced by CK2 inhibitor (Fig. 6A & B). Since IGFBP-3 fails to activate caspase-8, these effects are exerted through the extrinsic apoptotic pathway. In contrast, treatment of PC-3 cells with IGFBP-3 leads to the activation of caspase-8-mediated apoptosis (Fig. 6C), which is unaffected by incubation with either NU7026 (DNA-PK inhibitor) or TBB (CK2 inhibitor). These data suggest that there are at least two distinct mechanisms by which IGFBP-3 induces apoptosis: an intrinsic mechanism requiring nuclear localization and the subsequent interaction with RXRα, and a second, possibly independent pathway mediated by the extrinsic apoptotic machinery.

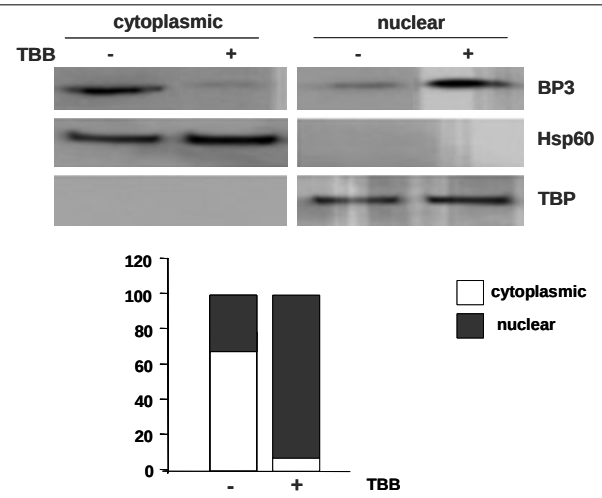

Figure 5 – Enhanced nuclear localization of IGFBP-3 in the absence of active CK2α. LAPC4 cells were incubated with 900 nM TBB for 24 h in SF media. The localization of endogenous IGFBP-3 was assessed by cellular fractionation followed by immunoblotting for IGFBP-3, Hsp60 (cytoplasmic control) and TBP (tata binding protein, nuclear control). Lower panel demonstrates cytoplasmic/nuclear localization of IGFBP-3 expressed as percentage of total IGFBP-3.

In order to identify the specific site in IGFBP-3 responsible for the enhanced apoptosis induction observed in the absence of active CK2, the two putative CK2 phosphorylation sites identified by bioinformatics (Fig. 2) in the central region of IGFBP-3 (S167, S175) were mutated to alanine by site directed mutagenesis. The resulting constructs were transfected in to LAPC4 and 22RV1 prostate cancer cells, and their ability to induce apoptosis, assessed by cleavage of a luminometric caspase-3/7 substrate, was compared with wild type IGFBP-3 48 h after

transfection. Transfection with either wtIGFBP-3 or IGFBP-3/S175A caused the induction of apoptosis which was significantly enhanced by incubation with 900 nM TBB (Fig. 7). In contrast, transfection with IGFBP-3/S167A caused an already enhanced apoptosis induction which was unaffected by the presence of a CK2 inhibitor, suggesting that S167 of IGFBP-3 may be one residue that can be phosphorylated directly by CK2, and that this phosphorylation event limits the ability of IGFBP-3 to induce apoptosis.

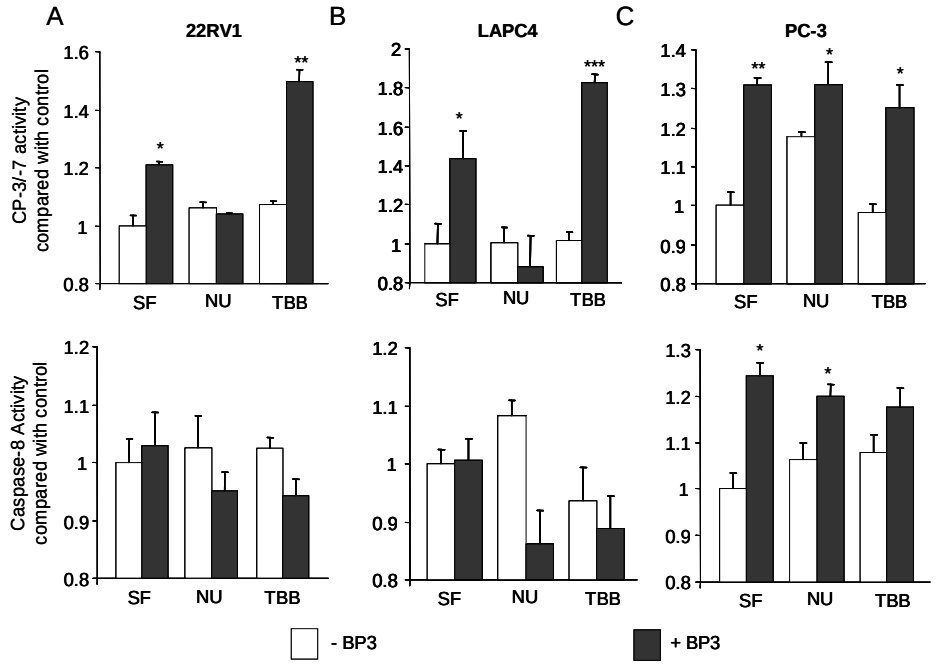

Figure 6 – DNA-PK and CK2 regulate IGFBP-3-induced apoptosis in 22RV1 and LAPC4, but not PC-3 cells. 22RV1 (A), LAPC4 (B) and PC-3 (C) cells were incubated for 24 h in the presence or absence of 1 μg/ml IGFBP-3 ± 10 μM NU7026 or 900 nM TBB. Apoptosis was assessed by cleavage of luminometric substrates for caspase-3/7 (upper panels) and caspase-8 (lower panels, as a measure of the extrinsic apoptotic pathway. Data are presented as means ± S.E.M. Significance that mean is different from 1: **P*<0.05, ***P*<0.01, ****P*<0.001.

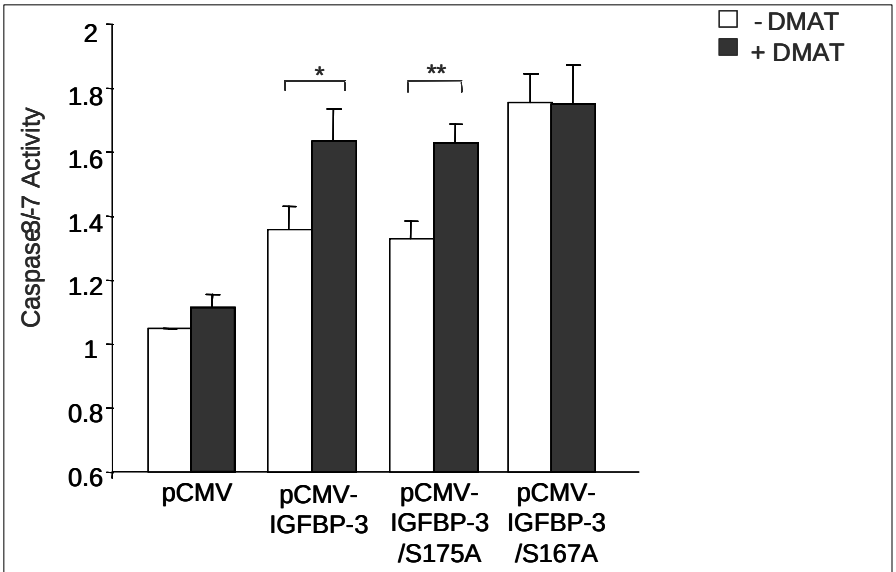

Figure 7 – IGFBP-3/S167A has enhanced ability to induce apoptosis and is unaffected by incubation with TBB. LAPC4 cells were transiently transfected with control vector (pCMV), wtIGFBP-3, IGFBP-3/S175A or IGFBP-3/S167A. 48 h after transfection, cells were transferred to serum free media ± 900 nM TBB. Apoptosis induction was assessed by cleavage of luminometric caspase-3/-7 substrate. Means ± S.E.M. Significance that mean is different from 1: * *P*<0.05; ** *P*<0.01

Since phosphorylation of IGFBP-3 by DNA-PK not only regulated apoptosis induction, but was also necessary for growth inhibition, we also compared the ability of wtIGFBP-3 and IGFBP-3/S167A to influence cell proliferation. LAPC4 cells were transfected with control vector, wtIGFBP-3 and IGFBP-3/S167A, and cell proliferation was assessed by MTT assay and BrdU incorporation ELISA. IGFBP-3 caused a 15-20% reduction in the growth of LAPC4 cells, as assessed by both methods, consistent with previous findings (Fig. 8). However IGFBP-3/S167A caused no further reduction in cell number/growth, suggesting that phosphorylation by CK2 at S167 is important for the regulation of the apoptotic actions of IGFBP-3, but not for its growth inhibitory actions.

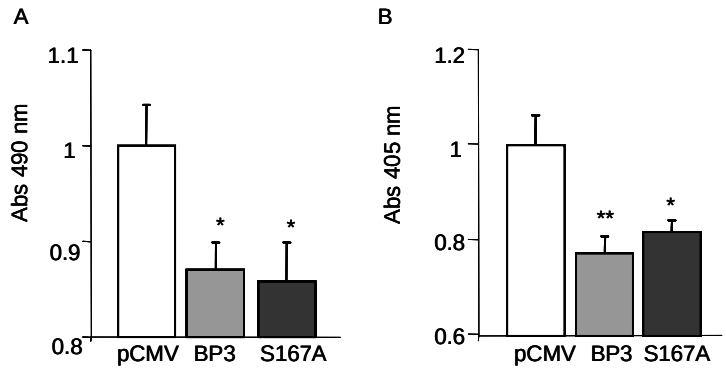

Figure 8 – **Phosphorylation of S167A does not affect growth inhibition by IGFBP-3**. LAPC4 cells were transiently transfected with control vector (pCMV), wtIGFBP-3 (BP3), or IGFBP-3/S167A (S167A). *A*, 48 h after transfection, cells were transferred to SF media for 72 h. Cell growth was assessed by MTT assay. *B*, cells were incubated in serum free media for 24 hours, and BrdU incorporation was assessed using ELISA assay. Means ± S.E.M. Significance that mean is different from 1 * $P<0.05$; ** $P<0.01$.

The data described so far have revealed two distinct phosphorylation events regulating IGFBP-3: a pro-apoptotic event occurring at S156, and an anti-apoptotic phosphorylation at S167. To determine whether the pro- or anti-apoptotic phosphorylation event is dominant, we utilized a combination of chemical inhibitors (NU7026 for DNA-PK and TBB for CK2) and our non-phosphorylateable mutants (S156 for DNA-PK and S167 for CK2), and compared the ability of IGFBP-3 to induce apoptosis under different conditions. As described, the transfection of LAPC4 or 22RV1 cells with wtIGFBP-3 causes the induction of apoptosis which is inhibited by NU7026 and enhanced by TBB (Fig. 9A). IGFBP-3/S165A is unable to induce apoptosis, and is completely unaffected by the presence of NU7026. However, in the presence of the CK2 inhibitor, IGFBP-3/S156A is able to induce apoptosis to the same extent as wtIGFBP-3, suggesting that preventing the activity of CK2 allows IGFBP-3 to overcome the inhibition caused by the absence of DNA-PK phosphorylation (Fig. 9A). However, incubation with NU7026 was unable to inhibit the enhanced apoptosis induced by IGFBP-3/S167A, suggesting that the oncogenic phosphorylation event of CK2 is dominant over the tumor-suppressive action of DNA-PK. To confirm these observations, we assessed the ability of IGFBP-3/S167A to induce

apoptosis in M059J cell, a glioblastoma cell line null for DNA-PK in which wtIGFBP-3 is unable to induce apoptosis. As previously demonstrated [7], IGFBP-3 induces apoptosis in M059K cells (which express active DNA-PK), but is ineffective in M059J cells, which lack the catalytic subunit of DNA-PK (Fig. 9B). In contrast, IGFBP-3/S167A is able to induce apoptosis in both cell lines, albeit with a slightly reduced effect in M059J.

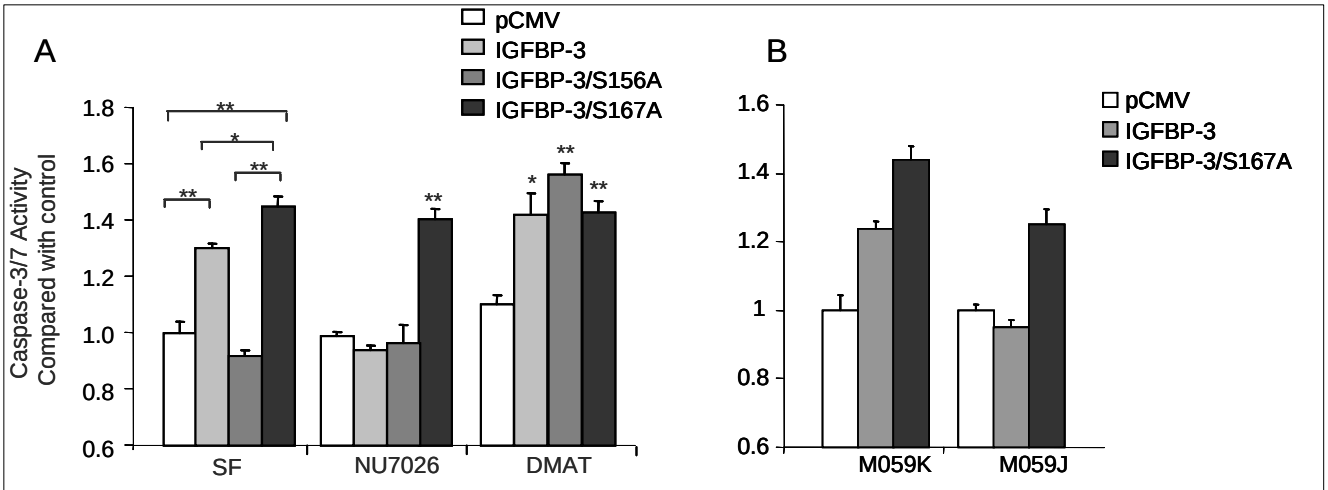

Figure 9 - Phosphorylation by CK2 is dominant over DNA-PK. *A*, LAPC4 cells were transfected with control vector (pCMV), wtIGFBP-3, IGFBP-3/S156A or IGFBP-3/S167A. 48 h after transfection, cells were transferred to serum free media in the presence or absence of 100 μM NU7026 or 900 nM TBB. Apoptosis induction was assessed after 24 h by cleavage of luminometric caspase-3/-7 substrate. *B,* Apoptosis was assessed by caspase-3/7 activity in M059K and J glioblastoma cells transiently transfected as in *A.* Means ± S.E.M. Significance that mean is different from 1: * $P<0.05$; ** $P<0.01$.

Together, these data provide a model whereby two distinct phosphorylation events in the central region of IGFBP-3 regulate its ability to induce apoptosis. The pro-apoptotic phosphorylation of

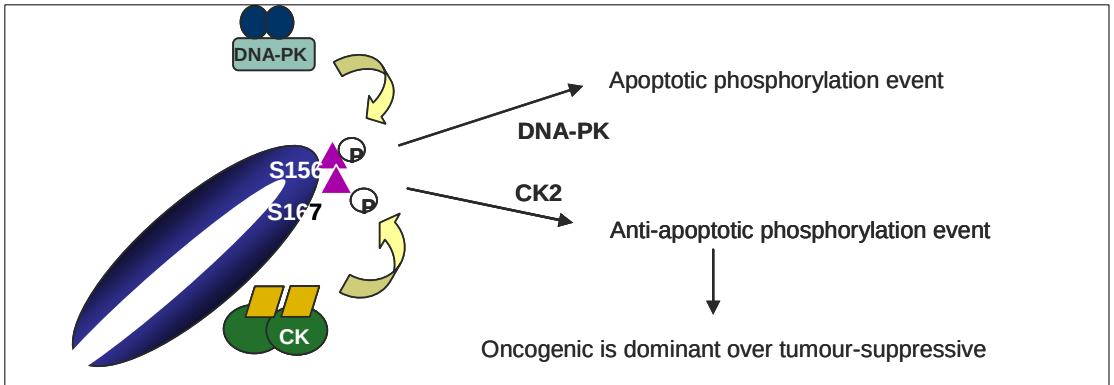

Figure 10 - Cartoon demonstrating the regulation of IGFBP-3 induced apoptosis by DNA-PK and CK2. Phosphorylation of S156 by DNA-PK acts as a pro-apoptotic event, allowing IGFBP-3 to induce apoptosis. Conversely, phosphorylation of S167 by CK2 is anti-apoptotic, limiting the ability of IGFBP-3 to induce apoptosis. The oncogenic phosphorylation event is dominant over the tumor-suppressive action.

S156 by DNA-PK is necessary for IGFBP-3-induced apoptosis in LAPC4 and 22RV1 prostate cancer cells. In contrast, the anti-apoptotic phosphorylation event by CK2 limits the ability of IGFBP-3 to induce apoptosis (Fig. 10).

Since we elucidated such a strong regulatory role for CK2 on IGFBP-3-induced apoptosis, and because CK2 is so commonly dysregulated in cancer, we decided to investigate whether the inhibition of IGFBP-3-induced apoptosis may be an important mechanism by which CK2 promotes cell survival. 22RV1 cells were transiently transfected with control (scrambled) or IGFBP-3 siRNA and incubated for 72 h. Media were then transferred to serum free in the presence or absence of high-dose (500 nM DMAT) CK2 inhibitor. In cells transfected with scrambled siRNA, treatment with high-dose inhibitor caused a significant induction of apoptosis, as assessed by ELISA for fragmentation of histone-associated DNA, which was unaffected by the simultaneous addition of exogenous IGFBP-3 (Fig. 11A). In contrast, DMAT was significantly less potent at inducing apoptosis when IGFBP-3 levels had been reduced by siRNA treatment. Indeed, treatment with exogenous IGFBP-3 restored apoptosis to levels comparable to scrambled siRNA-treated cells, suggesting that the presence of IGFBP-3 facilitates apoptosis induced by the inhibition of CK2.

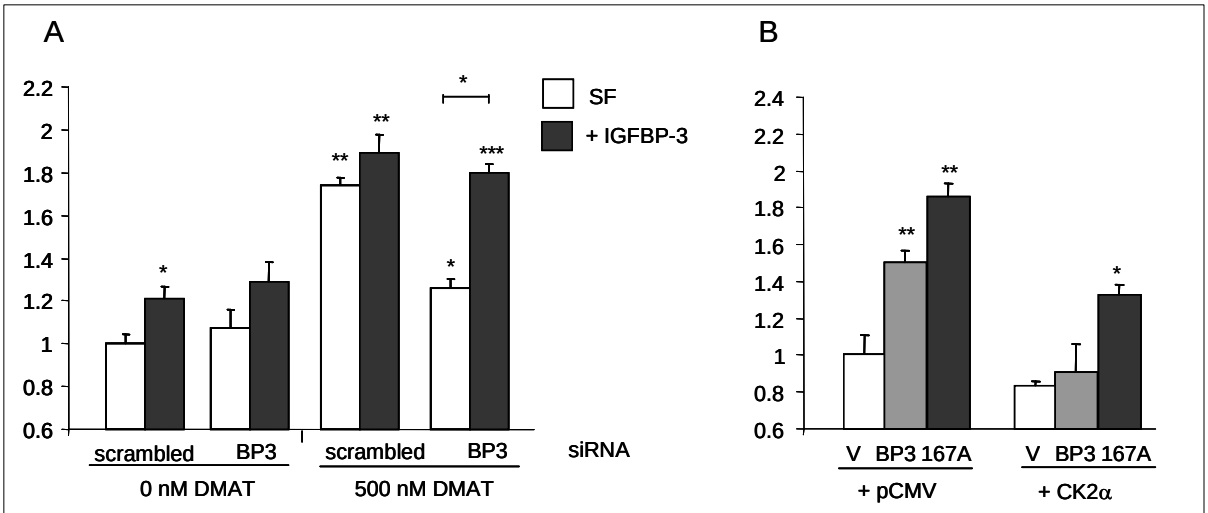

Figure 11 – Inhibition of IGFBP-3-induced apoptosis in one mechanism by which CK2 promotes cell survival. *A,* 22RV1 cells were transfected with scrambled or IGFBP-3 siRNA and incubated for 72 h. Histone-associated DNA fragmentation activity was assessed after 24 h incubated in SF media in the presence or absence of high dose (500 nM DMAT) CK2 inhibitor. *B,* 22RV1 cells were co-transfected with pCMV/pCMV-CK2α and pCMV, pCMV-IGFBP-3 or pCMV-IGFBP-3/S167A, and incubated for 48 h. Histone-associated DNA fragmentation activity was assessed after 24 h incubated in SF media. Means ± S.E.M. Significance that mean is different from 1: **P*<0.05; ***P*<0.01; ****P*<0.001.

To confirm these observations, we compared the ability of wtIGFBP-3 and IGFBP-3/S167A to induce apoptosis after over-expression of CK2α. 22RV1 cells were transfection with pCMV control vector, wtIGFBP-3 or IGFBP-3/S167A in combination with pCDNA3 or pCDNA3-CK2α. 48 h after transfection, media were transferred to SF, and apoptosis was assessed 24 h later by ELISA for fragmentation of histone-associated DNA. Consistent with previous data, IGFBP-3/S167A induces apoptosis more efficiently than wtIGFBP-3 when co-transfected with empty pcDNA3 vector. However, when CK2α is over-expressed, wtIGFBP-3 is unable to induce apoptosis, suggesting that it may be inactivated by the excess CK2. In contrast, IGFBP-3/S167A retains some ability to induce apoptosis. These data together suggest that the relationship and balance between CK2 and IGFBP-3 may play a key role in regulating apoptosis in prostate cancer cells.

Statement of work Task 3

The work defined by specific aim 3 of the research proposal was to create prostate specific IGFBP-3 transgenic mice, expressing wtIGFBP-3 and IGFBP-3/S156A, and to cross the resulting mice in to the TRAMP mouse model of prostate cancer. However, our laboratory is currently in the process of transferring from the TRAMP model (in which tumors develop very quickly) to the Myc model of prostate cancer, in which tumors have a slower development. The new model will allow investigation of the earlier stages of prostate cancer, and will be a better model for studying the therapeutic potential of different forms of IGFBP-3. Because of unforeseen circumstances with the characterization of the Myc model (unreliable genotyping, and the generation of high-level Myc expressors), the creation of the prostate-specific transgenics has not yet begun. Ultimately, the work following on from this fellowship grant will lead to the generation of inducible, prostate-specific transgenics not only of Myc-IGFBP-3 and Myc-IGFBP-3/S156A, but also Myc-IGFBP-3/S156E and Myc-IGFBP-3/S167A. The use of an inducible promoter will allow us to investigate the effectiveness of IGFBP-3 administration at different stages of cancer development, and will hopefully provide important insights in to the therapeutic potential of IGFBP-3 isoforms.

To study the potential importance of IGFBP-3 phosphorylation *in vivo* in the short-term, we therefore set up a series of xenograft experiments. 22RV1 xenograft tumors were generated by injection of 1 x 10^6 cells in 200 µL mixed at a 1:1 dilution with Matrigel in the right flank of male severe combined immunodeficiency mice. Tumors were established for 2 weeks before the start

of treatment. Tumors were treated daily by IP injection with 10 mg/kg/day IGFBP-3, 1 μM/day DMAT or a combination. The length and width of the mass located at the site of injection of the 22RV1 cells were measured with calipers and recorded once a week. The mice were euthanized at 10 days. Tumors were harvested, weighed, fixed in formaldehyde, and embedded in paraffin. Animal care was in accordance with current regulations and standards of the NIH, as well as our institutional guidelines for animal care.

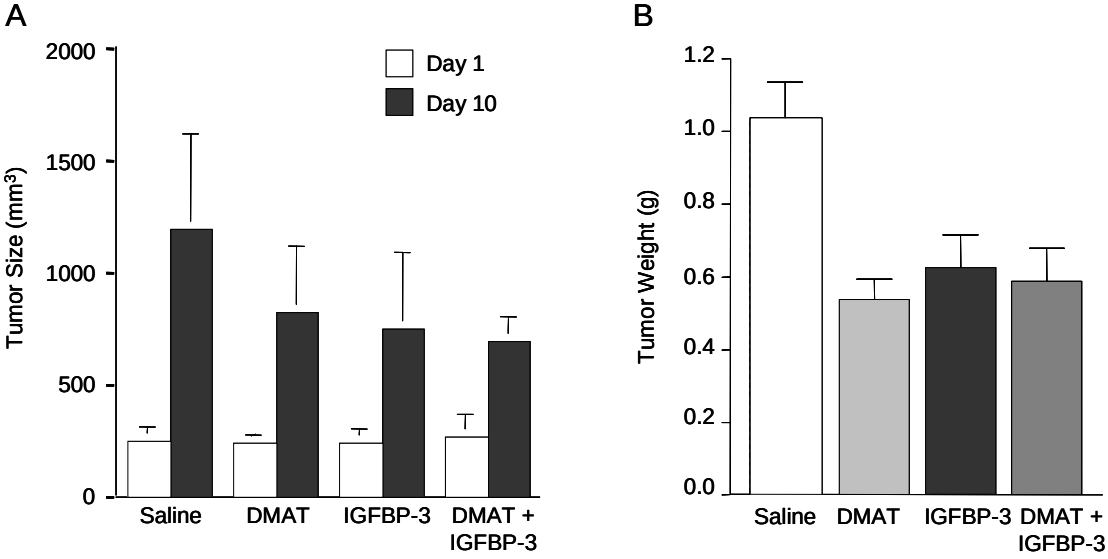

Figure 12 – Treatment with DMAT or IGFBP-3 inhibits tumor growth. The size and weight of 22RV1 xenograft tumors daily i.p. injected with saline, 10 mg/kg/day IGFBP-3, 1μM DMAT or combination for 10 days. *A,* caliper-measure tumor size and *B,* tumor weight at the time of sacrifice.

Both IGFBP-3 and DMAT individually exhibited a dramatic reduction of tumor size and volume (Fig. 12). However, combination therapy showed no either additive or synergistic effects of treatment. Tumor sections were also labeled with TUNEL as a measure of apoptosis (Fig. 13). Treatment with IGFBP-3 caused massive induction of apoptosis, although only at localized sites within the tumor. In contrast, DMAT treatment seemed to induce little apoptosis, suggesting that the reduced tumor size and weight observed was due to another factor, possibly toxicity-induced necrosis. Because of this, no additive or synergistic effects on apoptosis induction were observed with IGFBP-3 and DMAT co-treatment.

These observations suggest one of two things: either IGFBP-3 acts by distinct mechanisms in the tumor environment compared with an *in vitro* system; or, most likely a lower dose treatment with both IGFBP-3 and DMAT will be needed in order to observe the synergistic effect of tumor size and the induction of apoptosis. Indeed, previous experiments carried out in our laboratory

looking at the effects of i.p. injection of IGFBP-3 on tumor growth have demonstrated a 10-20%

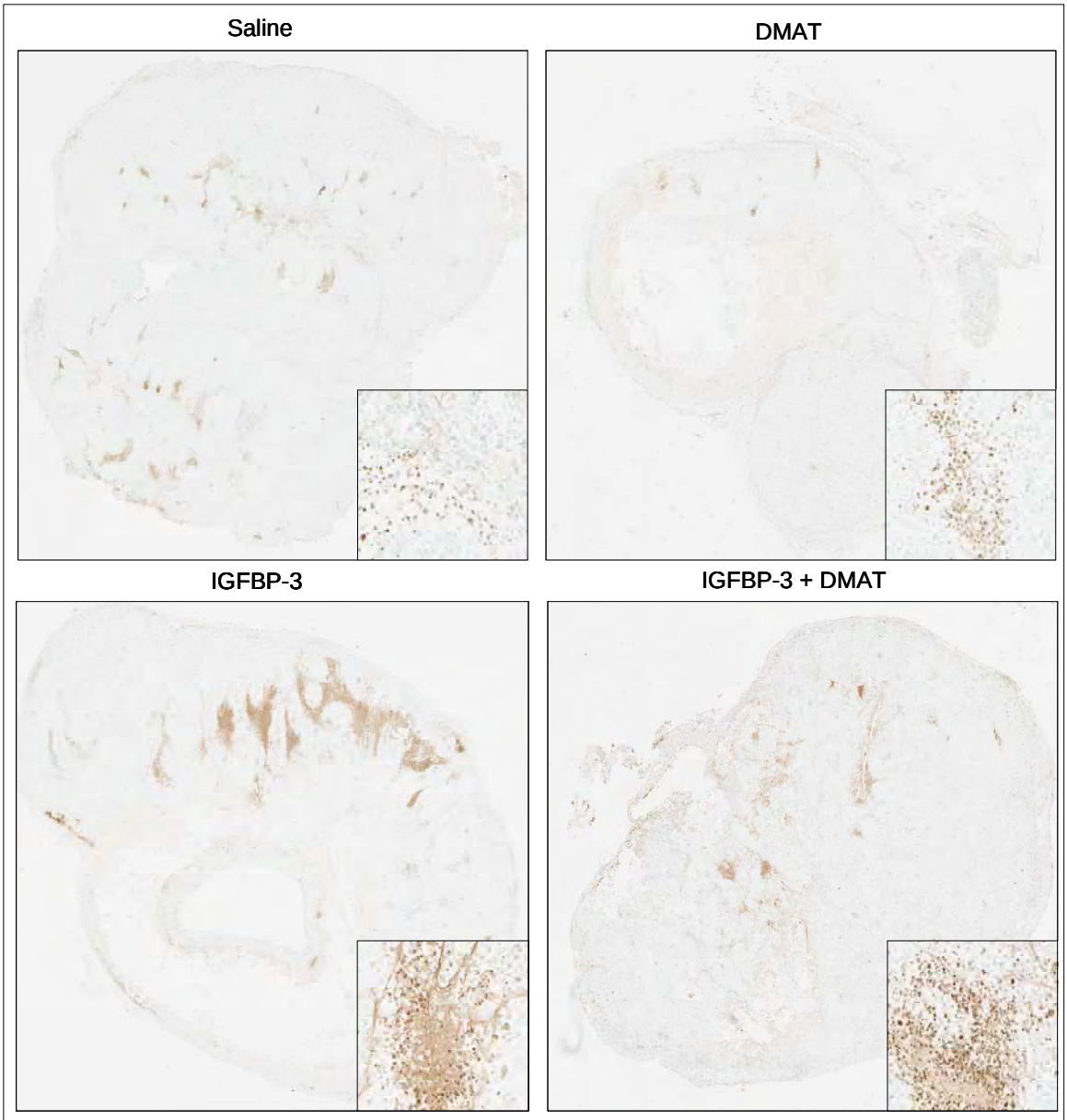

Figure 13 – Inhibition of tumor growth by IGFBP-3 is associated with increased apoptosis. Terminal nucleotidyl transferase–mediated nick end labeling (TUNEL) immunohistochemistry of xenografts, representative slides are shown.

reduction in tumor size. In the experiments presented here, we observed a 40% reduction, suggesting that the IGFBP-3 is far more potent, or possibly pure, than previous stocks. Since it is know that treatment with high dose CK2 inhibitor also exerts apoptosis, it will seemingly be necessary to also use a lower dose in future experiments.

Key Research Accomplishments

- Identification of phosphorylation by DNA-PK to be essential for IGFBP-3 mediated apoptosis
- Identification of Ser-156 as a critical residue for IGFBP-3-induced apoptosis
- Production of IGFBP-3/S156A, an expression vector of IGFBP-3 which is unable to induce apoptosis
- Identification of CK2 as an IGFBP-3 kinase, and discovery of S167 as the residue specifically phosphorylated
- Identification of IGFBP-3/S167A as an improved-potency apoptosis inducing protein, and production of the expression plasmid
- Recognition of phosphorylation as the dual-specificity mechanism for the regulation of IGFBP-3-induced apoptosis
- Identification that the relationship between CK2 and IGFBP-3 specifically may play a key role in regulating apoptosis in prostate cancer

Reportable Outcomes

- Cobb LJ, Liu B, Lee KW, Cohen P (2006). Phosphorylation by DNA-Dependent Protein Kinase is Critical for Apoptosis Induction by Insulin-Like Growth Factor Binding Protein-3. *Cancer Res*. 66: 10878-10884.

- Lee KW, Cobb LJ, Paharkova-Vatchkova V, Liu B, Milbrandt J, Cohen P (2007). Contribution of the orphan nuclear receptor Nur77 to the apoptotic action of IGFBP-3. *Carcinogenesis* 28: 1653-8.

- Anzo M, Cobb LJ, Hwang D, Mehta H, Said J, Yakar S, LeRoith D and Cohen P. Deletion of hepatic IGF-1 gene in TRAMP mice leads to dramatic alterations in the circulating IGF axis but does not reduce tumor progression. Accepted with revision to *Cancer Research*

- Abstract, ENDO 2006 (poster presentation). Phosphorylation of Ser-156 by DNA-PK is functionally critical for apoptosis induction by IGFBP-3. Laura J Cobb, Bingrong Liu, Kuk-Wha Lee and Pinchas Cohen.

- Abstract, 3rd GRS/IGF joint symposium (selected for oral presentation). Site specific phosphorylation by intracellular kinases determines the apoptotic activity of IGFBP-3. Laura J Cobb, Bingrong Liu, Kuk-Wha Lee and Pinchas Cohen

- Abstract, Gordon Research Conference IGFs in physiology and disease 2007 (poster presentation). Site-Specific Phosphorylation by Intracellular Kinases Determines the Apoptotic Activity of IGFBP-3. Laura J Cobb, Bingrong Liu, Satomi Koyama and Pinchas Cohen

- Abstract, Gordon Research Conference "IGFs in Physiology and Disease" 2007 (poster presentation). Synergism of IGFBP-3 and pomegranate extract to induce apoptosis in human prostate cancer cells. Satomi Koyama, Laura Cobb, Navindra Seeram, David Heber and Pinchas Cohen

- Abstract, American Association of Cancer Research Annual Meeting 2007 (poster presentation). Site-specific phosphorylation by intracellular kinases determines the apoptotic activity of IGFBP-3. Laura J Cobb, Satomi Koyama and Pinchas Cohen.

- Abstract, ENDO 2007 (poster presentation). Site-Specific Phosphorylation by Intracellular Kinases Determines the Apoptotic Activity of IGFBP-3. Laura J Cobb, Bingrong Liu, Satomi Koyama and Pinchas Cohen.

- Abstract, ENDO 2007 (poster presentation). Synergistic induction of apoptosis by IGFBP-3 and pomegranate extract in human prostate cancer cells**.** Satomi Koyama, Laura Cobb, Navindra Seeram, David Heber and Pinchas Cohen

- Abstract, ENDO 2007 (poster presentation). IGFBP-3 Inhibits Adipocyte Differentiation. John Ching, Hae-Soon Kim, Kuk-Wha Lee, Laura J Cobb and Pinchas Cohen

- Abstract, Specialized Programs of Research Excellence (SPORE) Meeting 2007 (poster presentation). Expression of the Novel Survival Peptide, Humanin, Is Associated with Prostate Cancer Recurrence. Pinchas Cohen, Laura J Cobb, Bingrong Liu, David Hwang and David Seligson.

- Abstract, Innovative Minds in Prostate Cancer Today Meeting 2007 (poster presentation). Enhancing the Apoptotic Potential of IGFBP-3 in Prostate Cancer by Regulation of Phosphorylation. Laura J Cobb, Bingrong Liu, Hemal Mehta and Pinchas Cohen.

- Abstract, Innovative Minds in Prostate Cancer Today Meeting 2007 (poster presentation). Expression of the Novel Survival Peptide, Humanin, Is Associated with Prostate Cancer Recurrence. Pinchas Cohen, Laura J Cobb, Bingrong Liu, David Hwang and David Seligson.

- IGFBP-3/S156A, an expression plasmid developed of a form of IGFBP-3 unable to induce apoptosis

- IGFBP-3/S167A, an expression plasmid developed of IGFBP-3 with increased potential to induce apoptosis

- Employment/research opportunities: Promotion to adjunct assistant professor at UCLA to continue these studies

- Funding applied for:

    - UCLA SPORE in prostate cancer career development award (in progress)
    - AACR career development award (in progress)

Conclusions

The IGF axis is known to play an important role in the epidemiology of many tumors, including prostate, lung and breast cancers. IGFBP-3 promotes apoptosis in cancer cells by both IGF-dependent and -independent mechanisms. We have previously shown that IGFBP-3 is rapidly internalized and localized to the nucleus, where its interactions with the nuclear receptor RXRα are important in apoptosis induction. Proteomic and bioinformatic analysis of IGFBP-3 reveals multiple consensus phosphorylation sites for kinases including CK2, PKA, PKC and cdc2. We have previously reported that phosphorylation of IGFBP-3 (S156) by DNA-PK enhances its nuclear accumulation, and is essential for its ability to interact with RXR and induce apoptosis in cultured prostate cancer cells. Indeed, IGFBP-3-S156A is completely unable to induce apoptosis in 22RV1 prostate cancer cells. Using specific chemical inhibitors, we investigated the contribution of other protein kinases to the regulation of IGFBP-3-induced apoptosis. Preventing the activation of CK2 enhanced the apoptotic potential of IGFBP-3. Using web-based proteomics software, we mapped two potential CK2 phosphorylation sites in IGFBP-3: S167 and S175. These sites were mutated to Ala, and the resulting constructs were transfected in to LAPC4 and 22RV1 prostate cancer cells. WtIGFBP-3 and IGFBP-3-S175A induced apoptosis to a comparable extent; however, IGFBP-3-S167A was far more potently apoptosis-inducing. Interestingly, IGFBP-3-S167A was able to induce apoptosis even in the absence of active DNA-PK, and IGFBP-3-S156A was able to induce apoptosis when CK2 activity was inhibited chemically or by using siRNA. Together, these data reveal two key regulatory phosphorylation sites in the central region of IGFBP-3. Phosphorylation of S156 by DNA-PK promotes apoptosis, whilst phosphorylation of S167 by CK2 limits the ability of IGFBP-3 to induce apoptosis in prostate cancer. Interestingly, our data suggest that the anti-apoptotic phosphorylation event induced by CK2 is dominant. These studies reveal multi-site phosphorylation of IGFBP-3 that both positively and negatively regulate its apoptotic potential. Understanding such intrinsic regulation of IGFBP-3 action may enhance the development of potential cancer therapies.

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Phosphorylation by DNA-Dependent Protein Kinase Is Critical for Apoptosis Induction by Insulin-Like Growth Factor Binding Protein-3

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Abstract

Insulin-like growth factor (IGF) binding protein-3 (IGFBP-3) promotes apoptosis of cancer cells by both IGF-dependent and IGF-independent mechanisms. *In vitro* phosphorylation of IGFBP-3 by DNA-dependent protein kinase (DNA-PK) has been reported but with unknown functional relevance. Using a chemical inhibitor for DNA-PK in prostate cancer cells and a paired system of glioblastoma cell lines that either lack or express DNA-PK, we show that the apoptosis-promoting and growth-inhibitory actions of IGFBP-3 are completely abrogated in the absence of catalytically active DNA-PK. In the absence of DNA-PK activity, IGFBP-3 has reduced nuclear accumulation and is unable to bind its nuclear binding partner retinoid X receptor (RXR) α. We assessed the importance of the three potential DNA-PK phosphorylation sites in IGFBP-3 using PCR-based site-directed mutagenesis. When transfected into 22RV1 cells, IGFBP-3-S165A and IGFBP-3-T170A functioned in an identical manner to wild-type IGFBP-3 to induce apoptosis. In contrast, IGFBP-3-S156A was unable to promote apoptosis and exhibited reduced nuclear accumulation, suggesting a key role for DNA-PK-dependent phosphorylation in the regulation of IGFBP-3 action. These studies reveal a novel regulatory mechanism for the actions of IGFBP-3 in prostate cancer and show phosphorylation of Ser^{156} to be functionally critical in its apoptosis-inducing actions. (Cancer Res 2006; 66(22): 10878-84)

Introduction

The activity of insulin-like growth factor (IGF)-I and IGF-II is regulated by a family of six high-affinity binding proteins. IGF binding protein (IGFBP)-3 is the most abundant of the IGFBPs in serum, where it forms a ternary complex with acid labile subunit and IGF (1). In addition to its role in regulating IGF action, IGFBP-3 exerts many IGF-independent effects to inhibit cell proliferation and enhance apoptosis in many cell types, including prostate (2) and breast (3–5) cancers.

IGFBP-3 has been reported in the nucleus of many cell types and contains a nuclear localization sequence (NLS) that facilitates nuclear uptake (6–8). Extracellular IGFBP-3 is rapidly internalized via transferrin receptor and caveolin and is transported into the nucleus by importin-β (9, 10). Once localized to the nucleus, IGFBP-3 interacts with the nuclear receptor retinoid X receptor (RXR) α to promote apoptosis by a mechanism that involves the nucleo-mitochondrial shuttling of RXRα/Nur77 (11, 12). However, IGFBP-3 may function in different ways to induce apoptosis because IGFBP-3 lacking a functional NLS is reported to promote apoptosis in breast cancer cells (13). However, little is understood about the cellular mechanisms regulating IGFBP-3 action.

IGFBP-3 is subject to post-translational modifications, such as glycosylation and proteolysis, and also contains consensus phosphorylation sites for a variety of protein kinases. In particular, Ser^{111} and Ser^{113} have been described as phosphoacceptor residues possibly for CK2 (14, 15). Phosphorylation of these sites may affect the ability of IGFBP-3 to become glycosylated because the S111A/S113A double mutant showed a strongly reduced glycosylation pattern (14). Phosphorylation of IGFBP-3 at the cell membrane of T-47D cells was reported to enhance IGF binding (16). IGFBP-3 can also be phosphorylated by DNA-dependent protein kinase (DNA-PK) and cyclic AMP–dependent protein kinase A (PKA) after incubation with recombinant enzyme and [γ-^{32}P]ATP (17). DNA-PK is a predominantly nuclear serine/threonine protein kinase, which is activated in response to DNA damage. It plays a role in numerous cellular processes, including DNA double-strand break repair, V(D)J recombination, telomere maintenance, and gene transcription (18). DNA-PK phosphorylates many transcription factors *in vitro*, including p53, a tumor suppressor that also functions to regulate the transcription of IGFBP-3 (19). Exogenously added IGFBP-3 that had been phosphorylated by DNA-PK displayed enhanced nuclear accumulation in Chinese hamster ovary (CHO) cells and decreased IGF binding compared with the nonphosphorylated form (17).

We investigated the significance of phosphorylation by DNA-PK for the cellular actions of IGFBP-3 in prostate cancer. We identify phosphorylation to be a critical step in the growth-inhibitory and apoptosis-promoting actions of IGFBP-3. DNA-PK-mediated phosphorylation enhances the nuclear accumulation of IGFBP-3 and is critical for interactions with its nuclear binding partner RXRα. Moreover, we reveal that Ser^{156} is the phosphoacceptor residue for DNA-PK and that this phosphorylation event is crucial for IGFBP-3 to exert these effects.

Materials and Methods

Reagents. Recombinant nonglycosylated IGFBP-3 was provided by Insmed (Glen Allen, VA). Goat anti-human IGFBP-3 antibody was purchased from Diagnostic Systems Laboratories (Webster, TX); rabbit anti-DYKDDDDK (FLAG tag) and rabbit anti-caspase-3 antibodies were from Cell Signaling Technology (Danvers, MA). The mouse anti-β-actin and mouse anti-Hsp60 antibodies, pCMV-FLAG expression vector, and the CelLytic NuCLEAR cell fractionation kit were purchased from Sigma (St. Louis, MO). Mouse anti-DNA-PK catalytic subunit antibody was from Kamiya (Seattle, WA). I-Block was purchased from Applied Biosystems

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(Foster City, CA). The rabbit anti-RXRα antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-TBP (nuclear loading control) antibody was purchased from Abcam (Cambridge, MA). Pfx DNA polymerase, T4 DNA ligase, LipofectAMINE 2000 transfection reagent, and all cell culture reagents were purchased from Invitrogen (Carlsbad, CA). NU7026, horseradish peroxidase–conjugated rabbit anti-goat, goat anti-rabbit, and goat anti-mouse secondary antibodies were from Calbiochem (San Diego, CA). The rabbit anti-phosphorylated serine/threonine antibody was from Chemicon (Temecula, CA). SDS-PAGE precast gels and blotting equipment were purchased from Bio-Rad (Hercules, CA). Restriction enzymes were from Fermentas (Hanover, MD). Recombinant DNA-PK and its substrate, ATP, CellTiter 96 AQueous One Solution Cell Proliferation Assay, and Apo-ONE Homogeneous Caspase-3/7 Assay were purchased from Promega (Madison, WI).

Cloning and mutagenesis. The putative DNA-PK phosphorylation sites of IGFBP-3 (Ser^{156}, Ser^{165}, and Thr^{170}) have been previously described (17) and were confirmed using NetPhos program in the CBS prediction servers (20). The three putative phosphorylation sites were individually mutated to alanine to prevent their phosphorylation. IGFBP-3 in PBS was mutated using PCR-based mutagenesis (sense primers: S156A, 5′-AAGAAAGGGCATGCTAAAGACGCCCAGCGCTACAAAGTTGACTACGAGTCTCA-3′; S165A, 5′-AGCCAGCGCTACAAAGTTGACTACGAGGCTCAGAGCACAGATACCCAGAACTT-3′; T170A, 5′-AAAGTTGACTACGAGTCTCAGAGCACAGATGCCCAGAACTTCTCCTCCGAGTCCAA-3′ and their reverse complement antisense copies) using Pfx DNA polymerase. Template DNA was digested using *Dpn*I (Fermentas), and all constructs (termed BP3, 156A, 165A, and 170A) were cloned into pCMV-FLAG (Sigma).

***In vitro* phosphorylation assay.** IGFBP-3 was phosphorylated *in vitro* by DNA-PK in the presence of ATP following the manufacturer's instructions. Phosphorylated IGFBP-3 was then analyzed by SDS-PAGE followed by phospho-specific immunoblotting.

Cell culture. The LAPC4 prostate cancer cell line was a generous gift from Charles Sawyers (University of California at Los Angeles, Los Angeles, CA). LAPC4 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 10 nmol/L R1881 (Perkin-Elmer Life Sciences, Wellesley, MA). 22RV1 prostate carcinoma cell line [American Type Culture Collection (ATCC), Manassas, VA] was maintained in RPMI 1840 supplemented with 10% FBS and 1% penicillin/streptomycin. M059K and M059J glioblastoma cell lines (ATCC) were cultured in F-12/DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% nonessential amino acids. For individual experiments, cells were seeded at a final density of $1 \times 10^5/cm^2$ in 96-well, six-well, or 10-cm plates and grown to 80% confluence in a humidified atmosphere of 5% CO_2 at 37°C before treatment. All treatments were carried out as indicated in serum-free medium.

Transient transfection. Cells growing on six-well plates were transfected using LipofectAMINE 2000 following the manufacturer's instructions. Briefly, 4 μg DNA was diluted in serum-free medium and combined with LipofectAMINE transfection reagent. Complexes were applied to cells in culture and incubated for 24 to 48 hours before analysis.

Immunoblotting. Cell lysates containing 20 μg protein were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membrane (Bio-Rad). Membranes were blocked in 0.2% I-Block in PBS containing 0.1% Tween 20 for 3 hours at room temperature and then probed with the appropriate primary and secondary antibodies. Antibody-antigen complexes were visualized by Western Lightning Chemiluminescence reagents (Perkin-Elmer Life Sciences) and autoradiography.

Cell fractionation. Cells on 10-cm plastic dishes were treated as indicated. Nuclear and cytoplasmic fractions were harvested using CelLytic NuCLEAR cell fractionation kit following the manufacturer's instructions. Separated fractions were quantified and analyzed by SDS-PAGE. Validity of separation was determined by immunoblotting for TBP and Hsp60.

Immunoprecipitation. Cell lysate (50 μg) or conditioned medium (10 mL) was incubated with 5 μL goat anti-human IGFBP-3 antibody overnight at 4°C. Protein A-Sepharose (50 μL, 25%) was added and samples were incubated at 4°C for 1 hour. Bound protein was eluted in Laemmli sample buffer [60 mmol/L Tris (pH 6.8), 2% SDS, 10% glycerol, 0.1% bromphenol blue], and the phosphorylation status of IGFBP-3 was assessed by immunoblotting with phosphorylated-specific antibodies. For coimmunoprecipitation experiments, samples were immunoprecipitated as above and analyzed by SDS-PAGE followed by immunoblotting.

Analysis of apoptosis. Apoptosis was assessed in cells growing in 96-well plates using Apo-ONE Homogeneous Caspase-3/7 Assay following the manufacturer's instructions.

3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium cell proliferation assay. To assess cell viability/proliferation, cells growing in 96-well plates were treated as appropriate and analyzed by CellTiter 96 AQueous One Solution Cell Proliferation Assay following the manufacturer's instructions.

Statistical analysis. Statistical analyses were analyzed using Student's t test and are presented as mean ± SE. Differences were considered statistically significant when $P < 0.05$.

Results

IGFBP-3 is phosphorylated by DNA-PK *in vitro*. It has been reported that adenoviral-derived IGFBP-3 can be phosphorylated by DNA-PK in the presence of [γ-^{32}P]ATP or using HeLa cell extract in the presence of a DNA-PK-specific substrate (17). To confirm our ability to detect *in vitro* DNA-PK-phosphorylated IGFBP-3, we incubated recombinant DNA-PK with recombinant IGFBP-3 in the presence of ATP. The resulting proteins were separated by SDS-PAGE and analyzed by phosphorylated-specific serine/threonine and IGFBP-3 antibodies. Phosphorylated-specific (serine/threonine) antibodies recognize IGFBP-3 only after incubation with DNA-PK, confirming that DNA-PK phosphorylates IGFBP-3 and that generic phosphorylated antibodies can be used to detect phosphorylated IGFBP-3 (Fig. 1*A*). We set out to confirm the phosphorylation of IGFBP-3 by DNA-PK *in vitro* using two systems. First, 22RV1 prostate cancer cells were incubated with 2 μg/mL IGFBP-3 in the presence and absence of 10 μmol/L NU7026, a specific ATP-competitive inhibitor for DNA-PK cells. IGFBP-3 was immunoprecipitated from cell lysates, and its phosphorylation status was analyzed by phosphorylated-specific immunoblotting. In the presence of NU7026, serine/threonine phosphorylation of both exogenously added (nonglycosylated, 29 kDa) and endogenous IGFBP-3 (glycosylated, 44 kDa) was reduced >3-fold (Fig. 1*B*). To confirm these observations, we used a paired cell system of glioblastoma cell lines that either lack (M059J) or express (M059K) DNA-PK (21). Endogenous IGFBP-3 was immunoprecipitated from M059K and M059J cells after 24 hours of incubation in serum-free medium in the presence or absence of NU7026, and phosphorylation was assessed using phosphorylated serine/threonine immunoblotting. Three-fold reduced phosphorylation of IGFBP-3 was observed in M059J cell lysates compared with M059K (Fig. 1*C*). In addition, phosphorylation of IGFBP-3 in M059K, but not M059J, cells was partially inhibited by coincubation with NU7026, confirming that IGFBP-3 is phosphorylated by DNA-PK *in vitro*. To determine whether secreted IGFBP-3 has been phosphorylated by DNA-PK, we incubated 22RV1 cells in serum-free medium in the presence or absence of NU7026 for 24 hours. IGFBP-3 was immunoprecipitated from conditioned medium, and its phosphorylation status was assessed by phosphorylated-specific immunoblotting. Similar amounts of both total and phosphorylated IGFBP-3 were detected in conditioned medium regardless of the presence of NU7026, suggesting that DNA-PK phosphorylation of IGFBP-3 does not occur during its secretion and that secreted IGFBP-3 does not get phosphorylated by DNA-PK in 22RV1 prostate cancer cells (Fig. 1*D*).

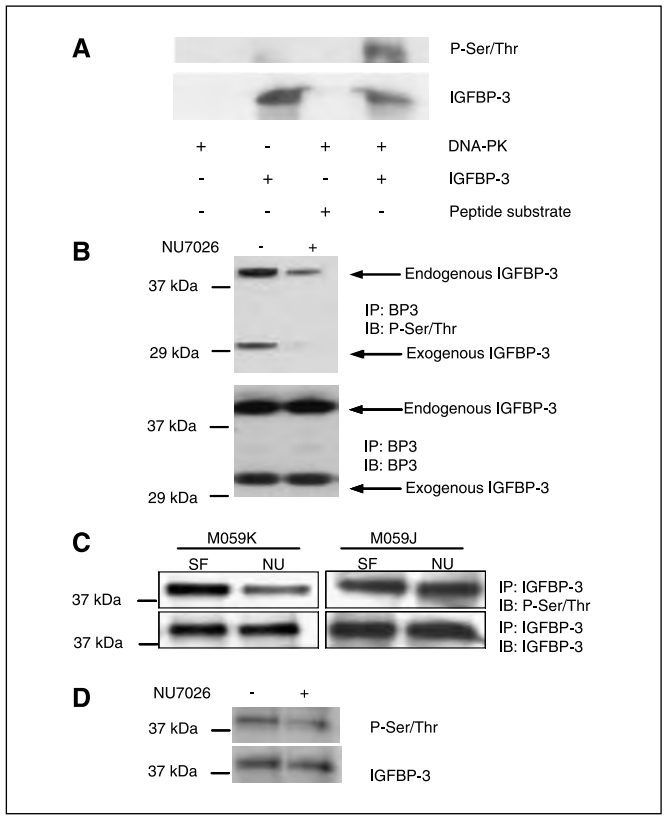

Figure 1. Reduced phosphorylation of IGFBP-3 in the absence of DNA-PK activity. *A,* recombinant DNA-PK was incubated with or without its peptide substrate or IGFBP-3 and ATP. Protein mixtures were separated by SDS-PAGE, and the phosphorylation status of IGFBP-3 was assessed by immunoblotting with phosphorylated serine/threonine (*P-Ser/Thr*) antibodies. *B,* 22RV1 cells were incubated in serum-free medium for 24 hours followed by 24 hours of treatment with 2 μg/mL IGFBP-3 and/or 10 μmol/L NU7026. The phosphorylation status of IGFBP-3 was assessed by immunoprecipitation (*IP*) with anti-IGFBP-3 followed by reducing SDS-PAGE and immunoblotting (*IB*) for phosphorylated serine/threonine (*top*) and IGFBP-3 (*bottom*). *C,* M059K and M059J glioblastoma cells were incubated in serum-free (*SF*) medium for 24 hours. Immunoblot for phosphorylated serine/threonine (*top*) and IGFBP-3 (*bottom*) after immunoprecipitation for IGFBP-3 followed by reducing SDS-PAGE. Each blot is representative of three independent experiments. *D,* phosphorylation status of IGFBP-3 secreted from 22RV1 cells incubated in serum-free medium in the presence or absence of NU7026 for 24 hours was assessed in conditioned medium as in (*B*).

Phosphorylation of IGFBP-3 by DNA-PK is necessary for its growth-inhibitory and apoptosis-inducing actions. IGFBP-3 directly inhibits proliferation (22, 23) and induces cell death in many tumor cell types, including prostate, lung, colon, and breast cancers (2, 3, 24, 25). Many post-translational modifications of IGFBP-3 have been reported, including phosphorylation by kinases, such as DNA-PK (15, 17). However, little physiologic relevance for such modifications is understood. Potential effects of DNA-PK activity on the ability of IGFBP-3 to inhibit cell growth were investigated by incubating LAPC4 cells with increasing concentrations of IGFBP-3 (0, 1, 2, and 4 μg/mL) in the presence or absence of NU7026 for 72 hours in serum-free medium. Treatment of LAPC4 cells with IGFBP-3 significantly inhibited cell growth in a dose-dependent manner (Fig. 2). However, coincubation with NU7026 completely prevented the growth-inhibitory actions of IGFBP-3. This suggests that phosphorylation of IGFBP-3 by DNA-PK is essential for its antiproliferative actions in prostate cancer. To determine whether phosphorylation of IGFBP-3 by DNA-PK plays a role in enhancing or inhibiting its apoptotic actions, we incubated 22RV1 and LAPC4 prostate cancer cells with 2 μg/mL human recombinant nonglycosylated IGFBP-3 ± 10 μmol/L NU7026 and assessed apoptosis induction using a fluorogenic caspase-3/caspase-7 substrate. In both cell types, treatment with exogenous IGFBP-3 caused a 40% increase in cleavage of caspase substrate compared with serum-free control ($P < 0.05$ in LAPC4; $P < 0.01$ in 22RV1; Fig. 3*A*). However, when cells were incubated with IGFBP-3 in the presence of the DNA-PK inhibitor NU7026, apoptosis induction by IGFBP-3 was completely abrogated and levels of caspase substrate cleavage were comparable with control cells. Incubation of either LAPC4 or 22RV1 cells with NU7026 alone caused no increase in caspase activity compared with serum-free controls, suggesting that NU7026 inhibits apoptosis induction by IGFBP-3 directly by inhibiting its phosphorylation as opposed to an indirect cellular effect.

To confirm these findings, we assessed caspase-3/caspase-7 activity in M059K and M059J cells incubated in the presence and absence of 2 μg/mL IGFBP-3 for 24 hours. The addition of IGFBP-3 to M059K cells led to a 30% increase in caspase activation ($P < 0.05$; Fig. 3*B*). In contrast, M059J cells, which completely lack the catalytic subunit of DNA-PK, have no significant response to treatment with IGFBP-3 (Fig. 3*B*). Taken together, these data suggest that DNA-PK activity is essential for the growth-inhibitory and apoptosis-inducing actions of IGFBP-3.

Phosphorylation of IGFBP-3 by DNA-PK enhances nuclear accumulation and is essential for interactions with RXRα. We have previously shown that the apoptosis-inducing actions of IGFBP-3 require its internalization, nuclear localization, and interaction with the nuclear receptor RXRα (10, 11). Because phosphorylation of IGFBP-3 by DNA-PK is also essential for its apoptosis-inducing actions, we hypothesized that phosphorylation of IGFBP-3 is necessary for its interaction with RXRα. 22RV1 prostate cancer cells were incubated for 24 hours in serum-free medium in the presence or absence of NU7026, and cytoplasmic and nuclear fractions were isolated. IGFBP-3 immunoblotting showed impaired nuclear localization of IGFBP-3 after treatment with NU7026 (Fig. 4*A*). To determine whether impaired nuclear

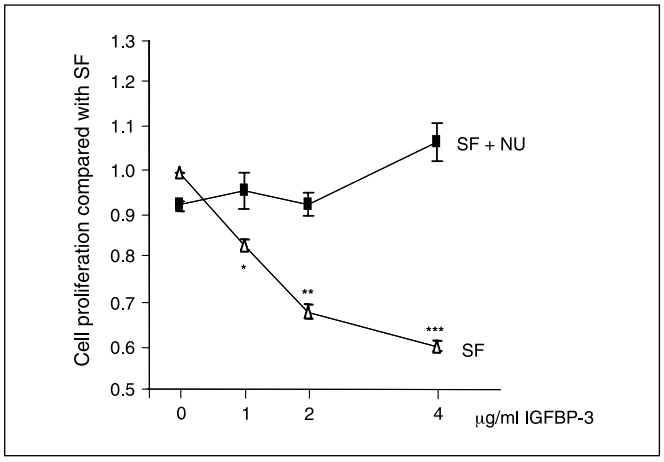

Figure 2. Growth inhibition by IGFBP-3 requires phosphorylation by DNA-PK. LAPC4 cells were incubated with increasing concentrations of exogenous IGFBP-3 for 72 hours in serum-free medium in the presence and absence of 10 μmol/L NU7026 (*NU*). Cell proliferation was assessed by enzymatic reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium to a formazan product. $n = 4$. Significance that mean is different from 1 (untreated control): *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

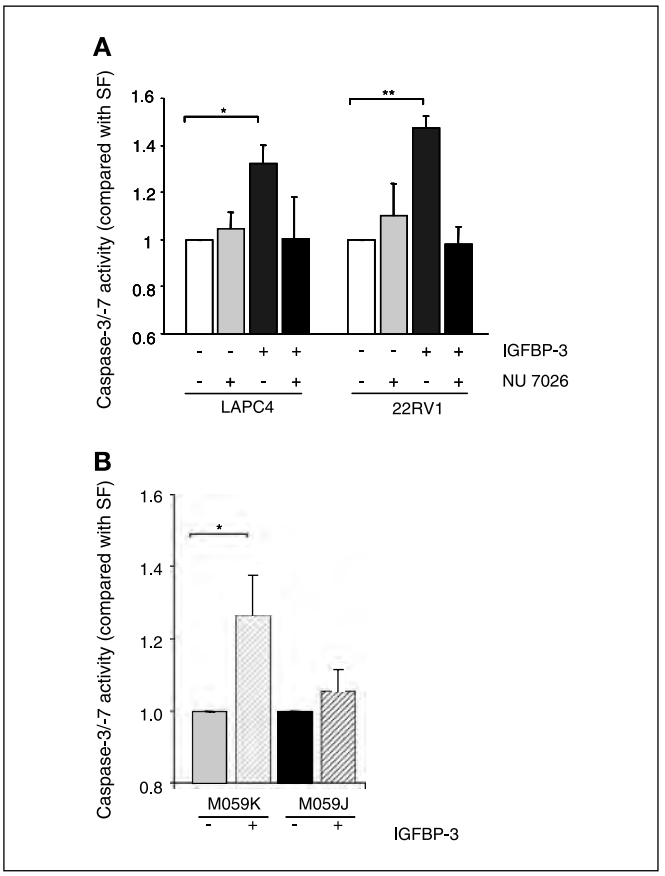

Figure 3. Apoptosis induction by IGFBP-3 requires phosphorylation by DNA-PK. *A,* LAPC4 and 22RV1 cells were incubated in serum-free medium for 24 hours followed by treatment with 2 μg/mL IGFBP-3 for 24 hours in the presence and absence of 10 μmol/L NU7026. Apoptosis was assessed by cleavage of a fluorogenic caspase-3/caspase-7 substrate. *B,* M059K and M059J cells were incubated in serum-free medium for 24 hours followed by treatment with 2 μg/mL IGFBP-3 for 24 hours. Apoptosis was measured as in (*A*). $n = 3$. Significance that mean is different from 1: *, $P < 0.05$; **, $P < 0.01$.

localization resulted in reduced RXRα binding, we incubated 22RV1 cells with 2 μg/mL IGFBP-3 in the presence and absence of 10 μmol/L NU7026 for 24 hours in serum-free medium and harvested whole-cell extracts. IGFBP-3 was immunoprecipitated from lysates and analyzed by SDS-PAGE. Coimmunoprecipitation of RXRα was detected by immunoblotting in cells incubated with IGFBP-3 alone (Fig. 4*B*). However, RXRα was no longer detected in the IGFBP-3 immunoprecipitation complex when cells were coincubated with IGFBP-3 and NU7026, correlated with reduced serine/threonine phosphorylation, suggesting that inhibiting the phosphorylation of IGFBP-3 by DNA-PK prevents its interaction with RXRα. These data were confirmed by assessing the ability of IGFBP-3 to interact with RXRα in M059K and M059J cell lines (Fig. 4*C*). When incubated with exogenous IGFBP-3, RXRα could be coimmunoprecipitated from M059K but not M059J cells, correlated with the phosphorylation status of IGFBP-3. When M059K cells were preincubated with NU7026, IGFBP-3 and RXRα no longer coimmunoprecipitated, confirming that DNA-PK activity is necessary for this interaction to occur. This provides a potential mechanism for the lack of apoptosis induction by IGFBP-3 observed in the absence of active DNA-PK.

Phosphorylation of Ser^{156} is critical for apoptosis induction by IGFBP-3. A cluster of three potential DNA-PK phosphorylation sites (Q/E/D-S/T-Q) have been identified in the central nonconserved domain of IGFBP-3, Ser^{156}, Ser^{165}, and Thr^{170}, which are highly conserved among human, mouse, rat, bovine, and porcine IGFBP-3 (17, 26). To determine the contribution of each residue to the functional regulation of the apoptotic actions of IGFBP-3, we mutated each residue individually to alanine by PCR-based site-directed mutagenesis. We then assessed the ability of pCMV-IGFBP-3-FLAG, pCMV-IGFBP-3/S156A-FLAG, pCMV-IGFBP-3/S165A-FLAG, and pCMV-IGFBP-3/T170A-FLAG expression to induce apoptosis in LAPC4 and 22RV1 prostate cancer cells. The expression of transfected constructs was verified by IGFBP-3 immunoblotting (Fig. 5*A*). Equivalent levels of endogenous IGFBP-3 (lower band) were detected in control cell lysates and in cells transfected with IGFBP-3. Slightly higher molecular weight IGFBP-3 was detected in cell lysates transfected with all forms of IGFBP-3 but was absent in pCMV-FLAG control transfected cells, corresponding to FLAG-tagged transfected constructs. Equivalent expression of all forms of transfected IGFBP-3 was observed. We assessed the ability of IGFBP-3/S156A, IGFBP-3/S165A, and IGFBP-3/T170A to induce apoptosis compared with wild-type IGFBP-3 by evaluating caspase-3/caspase-7 activity in transfected LAPC4 cells. Transfection of wild-type IGFBP-3 caused a 60% increase in apoptosis compared with control transfected cells ($P < 0.01$) that was completely abrogated by incubation with 10 μmol/L NU7026 (Fig. 5*B*). Similarly, LAPC4 cells transfected with either

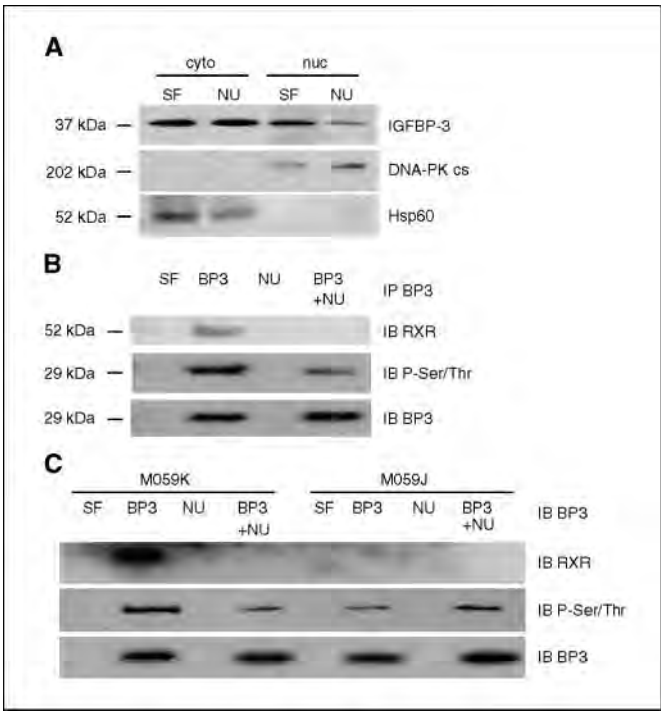

Figure 4. Preventing phosphorylation by DNA-PK reduces nuclear localization of IGFBP-3 and prevents interaction with RXRα. *A,* 22RV1 cells were incubated with and without 10 μmol/L NU7026 for 24 hours in serum-free medium. The intracellular localization of endogenous IGFBP-3 was assessed by anti-IGFBP-3 immunoblot after fractionation of nuclear (*nuc*) and cytoplasmic (*cyto*) fractions and SDS-PAGE. Validity of fractionation was confirmed by immunoblotting for Hsp60 (cytoplasmic fraction) and DNA-PKcs (nuclear fraction). The ability of IGFBP-3 to bind to RXRα in the absence of DNA-PK activity was assessed by immunoblotting for RXRα after immunoprecipitation with anti-IGFBP-3 in 22RV1 (*B*) or M059K/M059J (*C*) cells incubated in serum-free medium for 24 hours followed by treatment with 2 μg/mL IGFBP-3 for 24 hours in the presence and absence of 10 μmol/L NU7026. Blots are representative of three independent experiments.

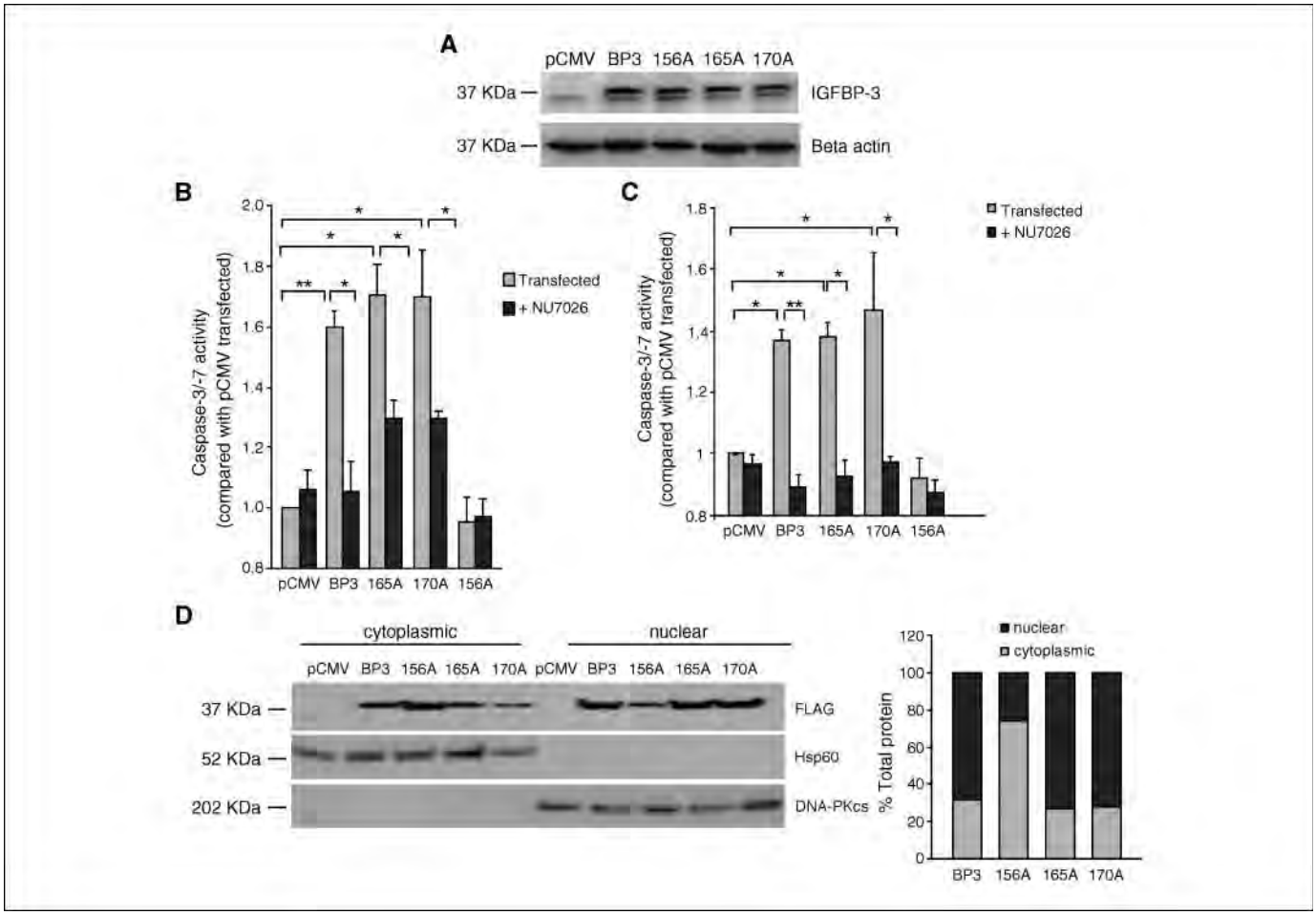

Figure 5. Phosphorylation of Ser^{156} is critical for apoptosis induction by IGFBP-3. Prostate cancer cells were transiently transfected with pCMV-FLAG (*pCMV*), pCMV-IGFBP-3-FLAG (*BP3*), pCMV-IGFBP-3/S156A-FLAG (*156A*), pCMV-IGFBP-3/S165A-FLAG (*165A*), or pCMV-IGFBP-3/T170A-FLAG (*170A*). *A,* immunoblot for IGFBP-3 (*top*) and β-actin (*bottom*, loading control) in 22RV1 cells transfected as above and harvested after 24 hours. Caspase-3/caspase-7 activity in LAPC4 (*B*) and 22RV1 (*C*) cells transfected as above and assayed after 24 hours. $n = 3$. Significance that mean is different from 1: *, $P < 0.05$; **, $P < 0.01$. *D,* 22RV1 cells were transfected as above and nuclear and cytoplasmic fractions were harvested after 24 hours. Immunoblot for FLAG (*top*), Hsp60 (*middle*, cytoplasmic control), and TBP (*bottom*, nuclear control). *Right,* quantification of FLAG immunoblot to show proportion of nuclear versus cytoplasmic protein.

pCMV-IGFBP-3/S165A or pCMV-IGFBP-3/T170A caused a 60% increase in caspase-3/caspase-7 activity that was also inhibited by coincubation with NU7026 ($P < 0.05$). In contrast, pCMV-IGFBP-3/S156A was unable to promote caspase activation either in the presence or in the absence of NU7026. To confirm these observations, we also analyzed caspase-3/caspase-7 activity in transfected 22RV1 cells (Fig. 5*C*). Cells transfected with wild-type IGFBP-3, IGFBP-3/S165A, or IGFBP-3/T170A displayed ~40% increased levels of caspase-3/caspase-7 activity compared with control transfected cells ($P < 0.01$ and $P < 0.05$, respectively). The increased caspase activation was inhibited by coincubation with NU7026. In contrast, 22RV1 cells overexpressing IGFBP-3/S156A displayed comparable levels of caspase activation with control cells and were unaffected by the addition of NU7026. Together, these studies suggest that phosphorylation of Ser^{156} by DNA-PK is essential for the apoptosis-inducing actions of IGFBP-3 in prostate cancer cells. Incubation of 22RV1 cells with NU7026 causes reduced nuclear localization of IGFBP-3 (Fig. 4*A*). To determine if reduced nuclear expression of IGFBP-3/S156A occurred, we transiently transfected 22RV1 cells with pCMV-FLAG, pCMV-IGFBP-3, pCMV-IGFBP-3/S156A, pCMV-IGFBP-3/S165A, and pCMV-IGFBP-3/T170A and isolated nuclear and cytoplasmic fractions. Cellular localization of transfected IGFBP-3 was assessed by SDS-PAGE followed by FLAG immunoblotting (Fig. 5*D*). Consistent with data obtained using NU7026 (Fig. 4*A*), we observed 3-fold reduced nuclear accumulation of IGFBP-3/S156A compared with wild-type IGFBP-3, IGFBP-3/S165A, and IGFBP-3/T170A. Validity of fractionation was confirmed by immunoblotting for Hsp60 (cytoplasmic fraction) and TBP (nuclear fraction). As expected, no FLAG immunoreactivity was detected in control transfected cell lysates. These data identify phosphorylation of Ser^{156} by DNA-PK as critical for the apoptotic actions of IGFBP-3 in prostate cancer cell lines.

Discussion

In addition to its role as the principal serum carrier of IGFs, IGFBP-3 also functions to potentiate and inhibit IGF action by regulating the bioavailability of IGFs to interact with the IGF type I receptor (1). In this way, IGFBP-3 can both ameliorate and abrogate IGF-stimulated cell proliferation and survival. Beyond its role of modulating IGF action, IGF-independent actions of IGFBP-3 have been described. For example IGFBP-3 is known to promote apoptosis in an IGF-independent manner in many cancer models

(2, 3, 24, 25). In addition, the absence of apoptosis in senescent fibroblasts has been associated with the absence of nuclear IGFBP-3 (27). Interestingly, human papillomavirus type 16 E7 oncoprotein, which can override senescence to immortalize human primary cells, can directly bind to and target IGFBP-3 for degradation (28). Several other cellular binding partners for IGFBP-3 have also been identified, including RXRα and humanin (11, 29, 30). Although factors, including p53, vitamin D, and transforming growth factor-β, are known to regulate IGFBP-3 expression (19, 31, 32), the mechanism of action for many IGF-independent roles of IGFBP-3 and how such functions are regulated are poorly understood.

Protein phosphorylation and dephosphorylation are common mechanisms for regulating the activity of numerous proteins and transcription factors in response to changing stimuli and environmental conditions (33). Of the six IGFBPs, phosphorylation has been reported for IGFBP-1 and IGFBP-3 (14–17, 34, 35). Previous reports of the effects of phosphorylation on IGFBP-3 action have suggested that post-translational modification of IGFBP-3 in this way may play a role in the regulation of IGF binding and nuclear localization (16, 17). Interestingly, phosphorylation has been reported to both enhance (16) and inhibit (17) IGF binding by IGFBP-3. Although these seem to be conflicting reports, it is possible that these are cell-specific effects or that different kinases play distinct roles in enhancing or preventing IGF-IGFBP binding. We have now identified phosphorylation of Ser^{156} of IGFBP-3 to be a critical step in the induction of apoptosis by IGFBP-3 in prostate cancer cells.

We have described the relevance of phosphorylation by DNA-PK for the roles of IGFBP-3 in prostate cancer. However, it is unclear what role phosphorylation by other kinases may play in regulating IGFBP-3 action. *In vitro* phosphorylation has been described by PKA and at residues consistent with consensus CK2 phosphorylation sites (14, 15, 17). Coverley et al. (15) showed an 80% decrease in [^{32}P]phosphate incorporation in CHO cells transfected with IGFBP-3 in which Ser^{111} and Ser^{113} (potential CK2 phosphoacceptor sites) had been mutated to alanine. In addition to suggesting that Ser^{111} and Ser^{113} can be phosphorylated, these data also suggested that other residues in IGFBP-3 are also phosphorylated. Similarly, comparing the phosphorylation status of IGFBP-3 in the presence and absence of active DNA-PK reveals partial but not complete reduction of phosphorylation without active DNA-PK, again suggesting that phosphorylation of IGFBP-3 by multiple kinases may occur. Although Ser^{111}/Ser^{113} phosphorylation may influence IGF binding by IGFBP-3, what significance phosphorylation by CK2, PKA, DNA-PK, and other unidentified kinases may have on other actions of IGFBP-3 is yet to be determined.

IGFBP-3 interacts with its nuclear partner RXRα to induce apoptosis in prostate cancer cells (11) in a nuclear localization–dependent manner. However, recent reports have revealed that IGFBP-3 is also able to induce apoptosis independent of nuclear localization. For example, a form of IGFBP-3 with a mutated NLS, which was unable to interact with the cell membrane and had impaired internalization, was still able to promote apoptosis in breast cancer cells (13). This suggests that IGFBP-3 is also able to promote apoptosis without being internalized [e.g., by interacting with a specific extracellular receptor (36)]. It is therefore possible that IGFBP-3 can function in different ways to promote cell death in cancer cells possibly in a cell type–specific manner.

Because DNA-PK is also predominantly a nuclear protein, it is likely that the phosphorylation of IGFBP-3 by DNA-PK occurs in the nucleus, promoting the association of IGFBP-3 with RXR and resulting in the induction of apoptosis. As Ser^{156} is in a region of IGFBP-3 distinct from the RXR-binding domain, it is possible that phosphorylation causes a conformational change in IGFBP-3 to facilitate interaction with RXRα. Such a mechanism would support data describing that nuclear localization is necessary for apoptosis induction by IGFBP-3 (11, 12). However, although DNA-PK acts predominantly as a nuclear kinase, low levels have been reported in cytoplasmic extracts derived from HTC rat hepatoma and HeLa cells (17, 37, 38), suggesting that phosphorylation of IGFBP-3 by DNA-PK may indeed occur in the cytoplasm or at the cell membrane. Indeed, phosphorylation of Akt by DNA-PK has been reported to occur at the cell membrane (39), suggesting that IGFBP-3 could potentially be phosphorylated during secretion or cellular uptake. However, our data suggest it is unlikely that phosphorylation by DNA-PK occurs during secretion in prostate cancer cells because there was little difference in phosphorylation status detected in IGFBP-3 from the conditioned medium of incubated with or without NU7026. However, phosphorylation by other kinases may occur either at the cell membrane or during secretion because phosphorylated IGFBP-3 is detectable in conditioned medium.

DNA-PK belongs to a family of large phosphatidylinositol 3-kinase-like proteins, which also includes ataxia-telangiectasia mutated and FRAP (40). Intracellular targets of DNA-PK kinase activity include p53, Mdm2, RNA polymerase II large subunit, and chromatin components (18, 41, 42). Functional DNA-PK consists of a catalytic subunit (DNA-PKcs) and a DNA-targeting heterodimer, Ku (43). Ku is tightly associated with DNA and functions by stimulating DNA-PKcs kinase activity toward DNA-bound targets and functions most effectively when the target protein is bound to the same DNA strand as DNA-PK itself (43, 44). Because DNA-PK requires DNA for its kinase activity, the demonstration of phosphorylation of IGFBP-3 by DNA-PK by ourselves and others would therefore seem to support growing evidence for either a direct or indirect role of IGFBP-3 in DNA binding and the modulation of gene transcription.

In summary, phosphorylation of IGFBP-3 by DNA-PK at Ser^{156} is a critical step in the cellular functions of IGFBP-3 in modulating apoptosis and growth inhibition. The generation of a novel nonphosphorylated mutant of IGFBP-3 will provide a crucial tool for future study of the biological actions of IGFBP-3.

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